Alcohol Dehydrogenase of Newly Isolated Furfuryl Alcohol Utilizing Strain

Kang, Sun Seon

Furfuryl Alcohol의 微生物學的 利用과 脫水素酵素

康 順 善

要 約

土壤에서 分離한 Furfuryl alcohol 資化性菌인 Cephalosporium屬에 依한 代謝및 重要한 性質에 對하여는 이미 報告한 바 있으며(1,2,3),

本報에서는 새로 分離된 Furfuryl alcohol 利用細菌 1001株의 脫水素酵素를 精製하고 純度 660倍 收率 9%로 電氣泳動的으로 均一한 標品을 얻어 그 性質를 調査하였다.

本酵素의 基質特異性을 酵母및 馬肝臟의 alcohol 脫水素酵素와 比較한 結果 Furfuryl alcohol및 다른alcohol에 基質特異性을 나타내었고 높은 活性을 가진 特徵的인 酵素였다.

또한 다른 alcohol 脫水素酵素와의 關連性에 對한 酵素蛋白進化論的 見地에서 檢討하였다.

Summary

A bacterial strain No. 1001 which has a high growth rate on a medium containing furfuryl alcohol as the carbon source was selected. The alcohol dehydrogenase of the strain was studied and the following results were obtained.

(1) Alcohol dehydrogenase of the strain was purified using cell extract of furfuryl alcohol grown cells as starting material. The enzyme was purified 660 times inspecific activity at a yield of 9% by chromatographies on DEAE-sephadex, Sephadex G-200, Blue Sepharose CL-6B and rechromatography on DEAE-Sephadex. The purified sample was homogeneous by electrophoresis.

(2) Alcohol dehydrogenase of strain 1001 had an optimum pH at 8.8-9.0 for furfuryl alcohol oxidation and an optimum temperature at 45°C.

(3) Kinetics of alcohol dehydrogenase of strain 1001 was studied for furfuryl alcohol and ethanol oxidation and their reverse reactions as an ordered Bi Bi reaction. V_m value for furfuryl alcohol oxidation was 38% of that of ethanol oxidation, but both substrates had almost same affinity to the enzyme. V_m values of reverse reactions, furfural and ethanol reductions, were almost same and furfural had rather higher affinity to the enzyme than acetaldehyde.

(4) The substrate specificity range of alcohol dehydrogenase of strain No. 1001 was compared to those of yeast and horse liver enzymes. Yeast alcohol dehydrogenase is characteristic to have a narrow substrate specificity and high activity and not active on furfuryl alcohol.

Horse liver enzyme which is active on furfuryl alcohol has a wide substrate specificity range and low catalytic activity. The alcohol dehydrogenase of strain No. 1001 had a wide substrate specificity including furfuryl alcohol and higher catalytic activity.

From these results, an evolution of substrate specificity range among alcohol dehydrogenases of various origin are discussed.

Introduction

In the previous reports, it was concluded that alcohol dehydrogenase is induced by furfuryl alcohol in Cephalosporium sp, the first step of furfuryl alcohol metabolism is catalyzed by alcohol dehydrogenase of wide specificity is active on furfuryl alcohol as well as primary alcohols and that no specific enzyme active on only furfuryl alcohol can detected. Alcohol dehydrogenases of various substrate specificity ranges have been studied, (29-31, 33, 34) and many of them are active on ethanol and other aliphatic alcohols with or without unsaturated aliphatic chain. Few enzymes are known to be active on furfuryl alcohol(28) or few informations are available on alcohol dehydrogenase action on furfuryl alcohol.

These obervations suggest that there might be wide varieties of alcohol dehydrogenases of which substrate specificities differ from species to species to species of microorganisms as a result of molecular evolution. In the case of other enzymes having absolute substrate specificity, the evolution of enzyme molecule can be followed by a change of amino acid sequence and causes alteration of catalytic activity and substrate specificity. But in case of alcohol dehydrogenase substrate specificity range would be altered, and such expanded substrate specificity might include; furfuryl alcohol which make the microorganism capable to metabolize furfuryl alcohol as the carbon source.

In this report another bacteria capable to grow on furfuryl alcohol medium was studied its alcohol dehydrogenase and compared the substrate specificity to that of Cephalosporium sp. yeast and horse liver.

Experimental Methods

Microorganism and Culture.

Bacteria No. 1001 strain which have been isolated from nature as a rapidly growing strain in furfuryl alcohol medium was used throughout this study.

For the bacterial growth, the basal medium contained 0.4g of NH_4NO_3, 2g of KH_2PO_4, 3g of K_2HPO_4, 0.2g of yeast extract, 0.1g of $MgSO_4 \cdot 7H_2O$, 10g of polypeptone and 3g of furfuryl alcohol in 1ℓ. Seed culture was incubated in 500mℓ of Ehlen-Meyer flask containing 100mℓ of the basal medium at 30℃ for 24 hours on a rotary shaker after inoculation from agar slant. Growth culture was carried out in a 3ℓ Sakaguchi flask containing 1,000mℓ of the basal medium, inoculated with 1% volume of the seed culture and incubated at 30℃ in a water bath with reciprocal shaking for 30 to 35 hours. During the incubation period more than 90% of furfuryl alcohol was consumed and the cell concentration reached to more than OD 9.

Cells were harvested by centrifugation in a refregirated centrifuge (KUBOTA Model KR-200A) and washed twice with buffer solution A. The buffer solution A was 0.02M potassium phosphate buffer pH 7 containing 10% glycerol and 10mM mercaptoethanol.

Protein assay

Protein assays were carried out by measuring the absorbance at 280nm using HITACHI spectrophotometer Model 200-20

Conductivity measurement

NaCl concentration was estimated by a conductivity meter of M & S Instrument Inc. Model CD-35MII.

Enzyme assay

The assay system contained in a final volume of 3.2mℓ : 50mM of Tris-HCl buffer pH 8.5, 0.1mM NAD^+ 1% furfuryl alcohol and 0.2mℓ of enzyme solution unless otherwise specified. The reaction was followed by measuring the increase in absorbance at 340nm by a HITAC

HI spectrophotometer Model 124 with a recorder.

Disc polyacrylamide gel electrophoresis

Disc polyacrylamide gel electrop horesis is carried our according to the method discribed by Davis (36) at pH 9.5. Separation gel contained 7% acrylamide in Tris-HCl buffer (pH 9) and the concentration gel contained 2.5% acrylamide in Tris-HCl buffer (pH 6.7). The pH of Tris-glycine buffer was pH 8.3. Electrophoresis was carried out at 4mA per tube. As a tracking dye bromophenol blue was used. After electrophoresis the gel was stained by amidoblack for one hour followed by destaining in 7% acetic acid at a constant current of 10 mA per tube.

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was adapted from Weber and Osborn(37). The gel contained 10% acrylamide monomer and 0.1% sodium dodecyl sulfate (SDS) in sodium phosphate buffer containing 0.1% SDS. Electrophoresis was carried out at 8mA per tube for 5 hours. The protein band was stained by amidoblack as stated above and destained under constant current (10mA per tube) in a solution containing 7% acetic acid and 5% methanol.

Reagents

DEAE-sephadex A-50, Sephadex G-200 and Blue Sepharose CL-6B were purchased from Pharmacia Fine Chemicals. Furfuryl alcohol was obtained from Nakarai Chemicals Co. and purified by fractional destillation and stored in N_2 atomosphere before use. Other alcohols and aldehydes were analytical grade of commercial products. Alcohol dohydrogenases of yeast and horse liver were products of Oriental Yeast Co. Ltd. and Sigma Chemical Co.

Coenzymes, NAD^+ and NADH were obtained from Oriental Yeast Co. and Kohjin Co. Ltd. NAD^+-N^6-[N-(N-acryloyl-1-methoxycarbonyl-5-aminopentyl)-propioamide] (M-NAD$^+$) and its acrylamide copolymer (P-NAD$^+$) were prepared by Muramatsu, Urabe, Yamada and Okada in Osaka University. Other chemicals were obtained commercially.

Results

Purification of Enzyme

Crude enzyme

Cells (7g) were suspended in 30ml of 0.02M potassium phosphate buffer containing 10% glycerol and 10mM mercaptoethanol (buffer A). The cells were disintegrated by French press(OTAKE WORKS Co.) at 400kg/cm² pressure and centrifuged at 12,100g for 10 minutes. The resulting pellet was again suspended and subjected to sonic treatmant at 20kc for 5 minutes to extract the enzyme. The resulting suspension was combined with the supernatant solution of the pressed juice and centrifuged in a ultracentrifuge apparatus of HITACHI 80P with a rotor RP50—2 at 50,000 rpm fof 45 minutes. The supernatant obtained was used as crude enzyme.

DEAE-Sephadex A-50 Column Chromatography

The crude enzyme solution obtained above was subjected to a column chromatography on DEAE-Sephadex A-50 which had been equilibriated with buffer A. The column size used was 3.0cm in diameter and 48cm long. After charge of the crude enzyme solution, enzyme was eluted by buffer A containing stepwisely increasing NaCl concentration; 0, 0.05, 0.1 and 0.15M NaCl at the elution rate 200ml per hour. Each 20ml fractions were collected. The result is shown in Fig. 1. The active enzyme fractions were collected (Fraction number 71 through 92) and concentrated in an Amicon filter UK 50 to 15ml. The active enzyme eluted at NaCl concentration of 0.1M.

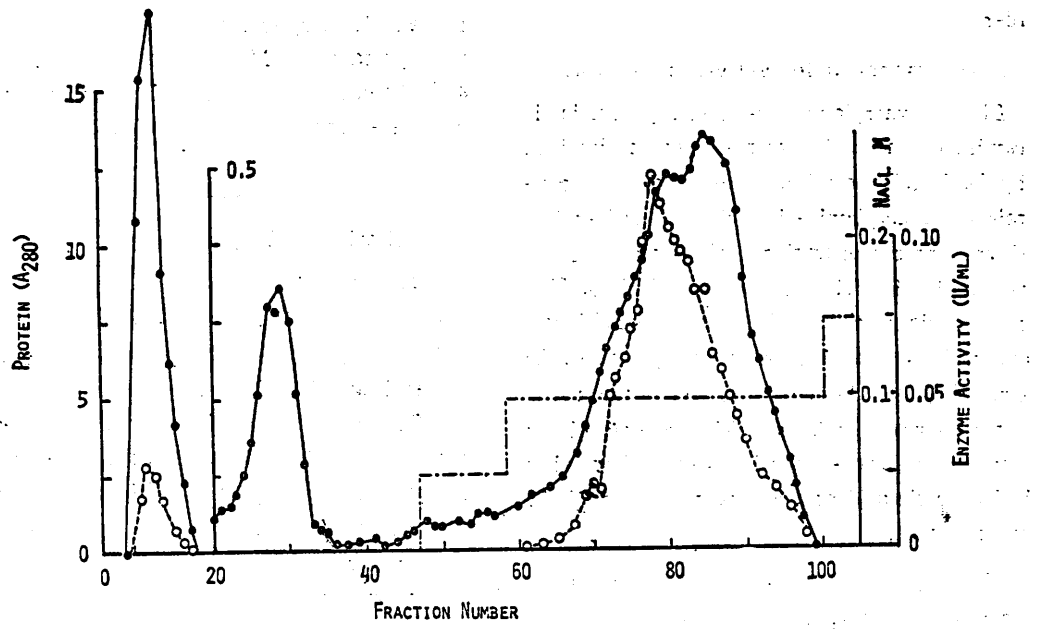

Fig. 1. Column chromatography of alcohol dehydrogenase of strain No. 1001 on DEAE Sephadex-A 50. Column size 3.0㎝ in diameter and 48㎝ long, and each fractions of 20㎖ was collected. Alcohol dehydrogenase Activity (○), Protein concentration (●).

Sephadex G-200 Column Chromatography

The enzyme concentrate obtained above was further puirfied on a column of Sephadex G-200, which had been equilibriated with buffer A. The column size used was 2.8㎝ indiameter and 52㎝ long. The enzyme was eluted by buffer A at an elution rate of 18㎖ per hour. and each 3㎖ fractions were collected. The chromatogram is shown in Fig. 2. The active fractions (Tube number 25 through 35) were collected and concentrated on an Amicon filter to 5㎖.

Affinity Chromatography on Blue-Sepharose CL-6B

The enzyme concentrate obtained by the molecular s hieve chromatography was subjected to an affinity chromatography on a column containing Blue-Sepharose CL-6B. The column (2㎝×13㎝) was equilibriated with buffer A, and after application of enzyme sample, the

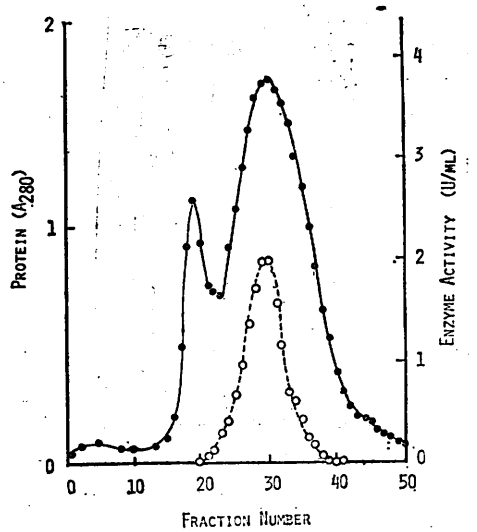

Fig. 2. Chromatography of alcohol dehydrogenase of strain No. 1001 on Sephadex G-200. Alcohol dehydrogenase activity (○), protein concentration (●).

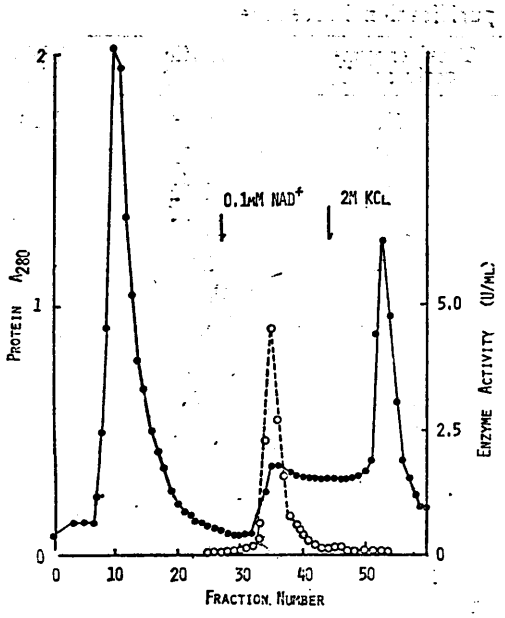

Fig. 3. Affinity chromatography of alcohol dehydrogenase of strain No. 1001 on Blue Sepharose CL-6B. The column size was 2cm in diameter and 13cm long. The eluting solution was buffer A containing specified reagents in the figure at the time indicated by arrows. Alcohol dehydrogenase activity (○), protein concentration (●).

column was eluted first with buffer A, then buffer A containing 0.1mM NAD^+ and then with buffer A containing 2M KCl. The elution rate was 15ml per hour and each 3ml fractions were collected. The results is shown in Fig. 3. The active fractions of tube number 33 through 37, were collected and again concentrated in an Amicon Filter.

Rechromatography on DEAE-Sephadex A-50 Column.

The enzyme sample obtained above was rechromatography on a column (1.5ml×15cm) containing DEAE-Sehadex which had been equiliated by buffer A. The column was eluted at a elution rate of 15ml per hour with buffer A. containing stepwisely increasing NaCl; 0.06, 0.08, 0.1, 0,12 and 0.15 M NaCl. The results is shown in Fig. 4. The active fractions (Tube number 51 through 60) were collected and concentrated until 3ml by an Amicon filter. The enzyme sample of this purity was used throughout this experiment.

The purification results are summarized in Table 1, which shows the alcohol dehydrogenase was purified about 660 times in specific activity yield 9%.

Purity of the Enzyme Sample.

As shown in Fig. 4, the activity peak fractions appeared in rechromatography on DEAE-Sephadex column had a constant specific activity. The purified enzyme sample showed a single band when analyzed either by disc acrylamide gel electrophoresis or by SDS gel electrophoresis as shown in Fig. 5.

Enzyme Characteristics.

Stability of enzyme

The purified enzyme is not stable at 4℃. It lose 50% activity in 24 hours in 0.02M phosphate buffer at pH 7.3 containing 10% glycerol. Addition of 10mM $NaHSO_3$, 30mM dithiothreitol or both did not improved the enzyme stability. The addition of 30mM 2-mercaptoethanol or 30mM mercaptoethanol and 30mM $NaHSO_3$ prevented the enzyme inactivation. At

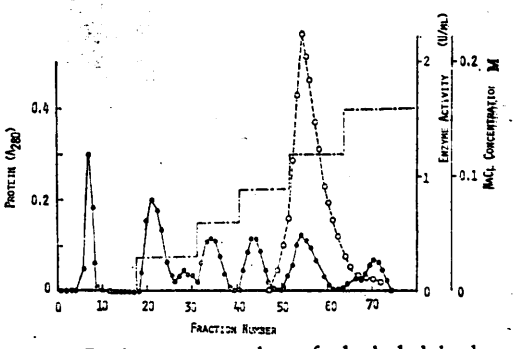

Fig. 4. Rechromatography of alcohol dehydrogenase on DEAE-Sephadex A50. Column size was 1.5cm in diameter and 15cm long. Each fraction of 2ml was collected. Alcohol dehydrogenase activity(○), protein concentration(●).

Table 1. Summary of purification Procedure

Purification step	Protein (㎎)	Total enzyme activity (U)	Specific (U/㎎)	Yield (%)
Crude enzyme	3,400	102	0.03	100
DEAE-Sephadex A-50	121	37	0.31	36
Sephadex G-200	39	36	0.92	35
Blue Sepharose CL-6B	1.2	14.8	12.55	15
DEAE-Sephadex A-50 rechromatography	0.46	9.2	20.0	9

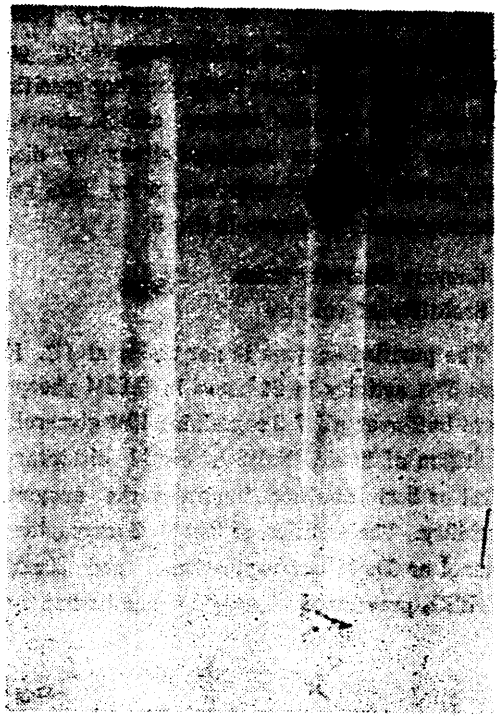

Fig. 5. Analytical disc gel and SDS gel electrophoregrams of alcohol dehydrogenase of strain No. 1001. Both gels were stained for protein using amidoblack solution.

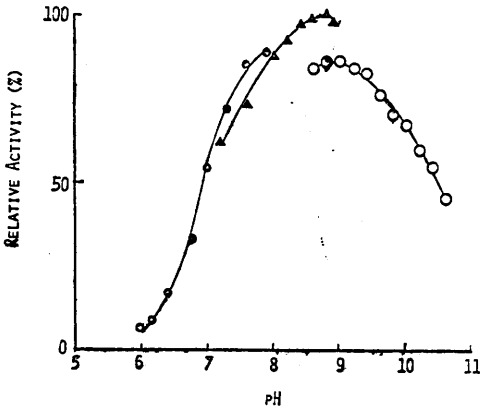

Fig. 6. Dependence of alcohol dehydrogenase activity of strain No. 1001 on pH. (●) Potassium phosphate buffer, (○) glycine-NaOH buffer, and (▲) Tris-HCl buffer. The reaction mixture contained 133mM buffer solution, 10mM mercaptoethanol, 100mM furfuryl alcohol and 0.1mM NAD^+ in a constant temperature(30℃) cell.

these conditions no detectable loss in 2 days and 10 to 20% loss in a week was observed.

Optimum pH

Optimum pH was determined to be 8.8 to 9.0 by using furfuryl alcohol as substrate. The buffer system used were each 0.13M potassium phosphate buffer for pH range of 5.7 to 7.9, Tris-HCl buffer for pH range of 7.2 to 8.9 and glycine-NaOH buffer for pH range of 8.6 to 10.6. The pH activity curve is shown in Fig. 6.

Optimum temperature

Optimum temperature of the dehydrogenase was determined to be 45℃ as shown in Fig. 7. The reaction rate was determined by NAD^+ reduction rate in the presence of furfuryl alcohol.

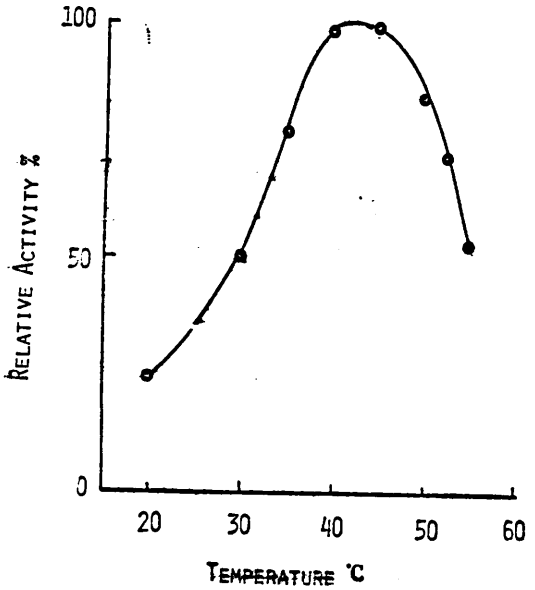

Fig. 7. Temperature-activity relationship. The reaction mixture was the same as legend of Fig. 6. except for 100mM Tris-HCl buffer of pH 8.5 was used through out.

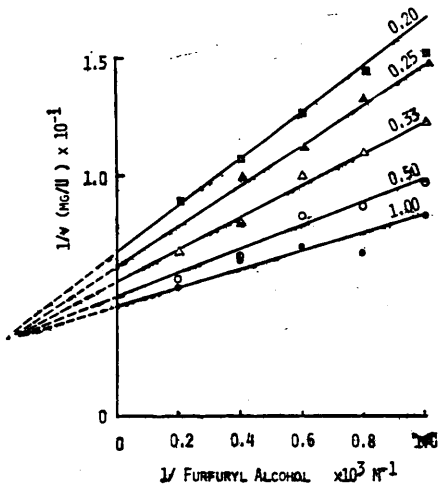

Fig. 8. Lineweaver-Burk plots for the oxidation of furfuryl alcohol with alcohol dehydrogenase of strain No. 1001. The coenzyme concentration was 0.20, 0.25, 0.33, 0.50 and 1.00 x10⁻⁴M as specified in the figure. Other reaction conditions were same as in legend of Fig. 7.

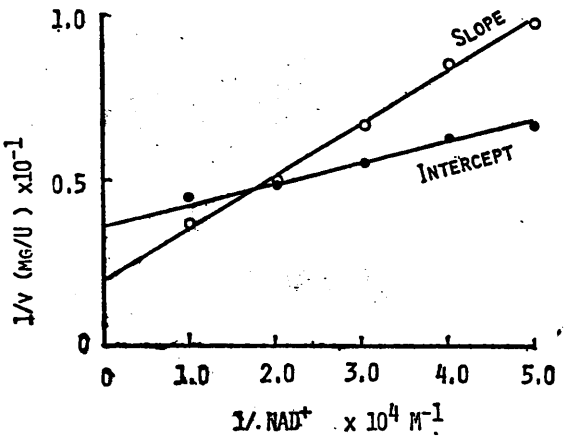

Fig. 9. Replots of the slope and intercepts of the Lineweaver-Burk plots shown in Fig. 8. against the reciprocal of NAD^+ concentration.

Kinetic properties.

Reaction sequence of alcohol dehydrogenase obeys mainly ordered Bi Bi reaction (38) where enzyme reacts first with NAD^+ molecule then react with alcohol to form tertial com plex, and among the reaction products, aldehyde releases from the enzyme molecule first then NADH.

Kinetic studies were carried out with reaction systems as described in "Materials and Methods". Kinetic constants in the initial rate equation for forward reaction.

$$\frac{V_{mf}}{v}=1+\frac{K_a}{[NAD^+]}+\frac{K_b}{[alcohol]}+\frac{K_{ia}K_b}{[NAD^+][alcohol]}$$

are obtained from Lineweaver-Burk plots and slope and intercept replots as described by Cleland (39). In the equation V_{mf} is the maximum velocity for forward reaction, K_{ia} the dissociation constant of the enzyme-NAD^+complex; K_a and K_b are the limiting Michaelis constants for NAD^+ and alcohol respectively. The results of initial rate studies with NAD^+ and furfuryl alcohol, and NAD^+ and ethanol are shown in the Lineweaver-Burk plots of

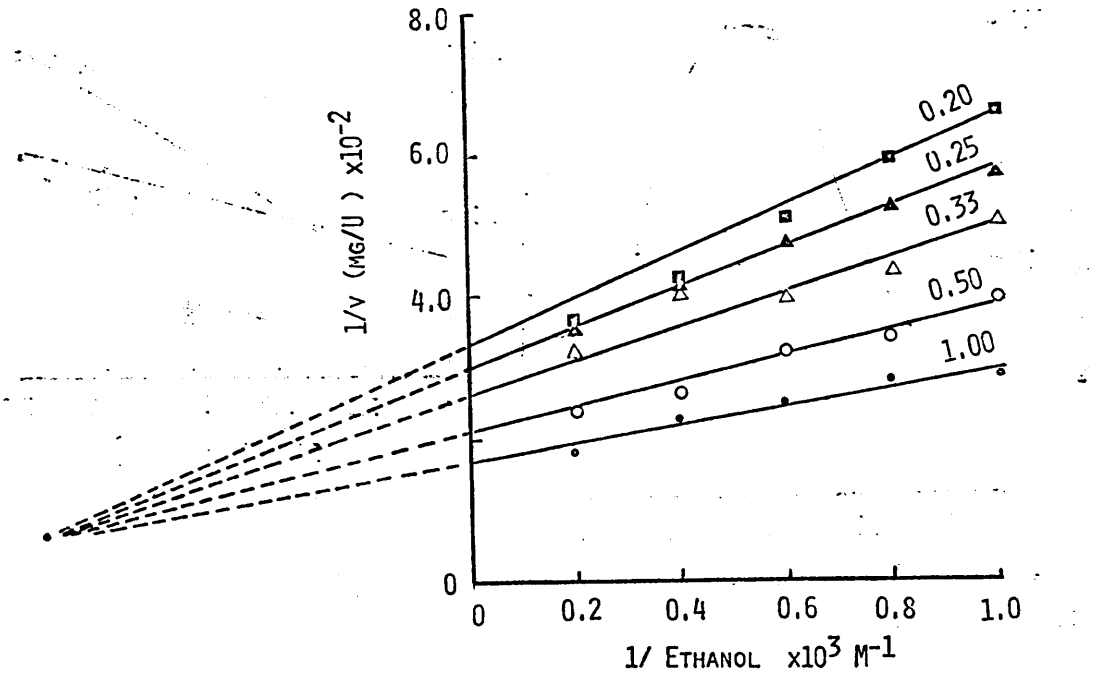

Fig. 10. Lineweaver-Burk plots for the oxidation of ethanol with alcohol dehydrogenase of strain No. 1001. The NAD^+ concentrations were 0.20, 0.25, 0.33, 0.50 and 1.00 $x10^{-4}$M.

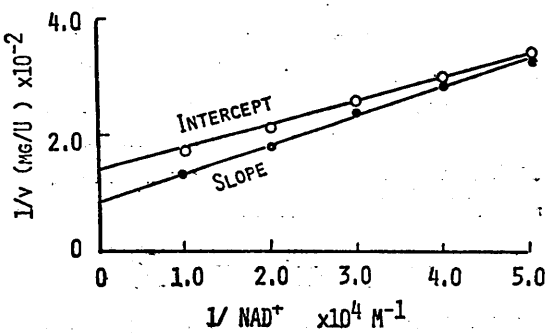

Fig. 11. Replots of the slopes and intercepts of the Lineweaver-Burk plots shown in Fig10. against the reciprocal of NAD^+ concentration.

Fig. 8. and 10 respectively. The slopes and intercepts replots of the results obtained in these figures are shown in Fig. 9 and 11 respectively.

The kinetic constants for the evrerse reactions are also studied by using furfural or acetaldehyde and NADH as substrate and coenzyme. The initial rate equation is as follows;

$$\frac{V_{mr}}{v}=1+\frac{K_p}{[\text{aldehydel}]}+\frac{K_q}{[\text{NADH}]}+\frac{K_{iq}\,K_p}{[\text{adehyde}][\text{NADH}]}$$

were V_{mr} is the maximum velocity of the reverse reaction, K_{iq} is the dissociation constant of enzyme-NADH complex; and K_p and K_q are also limiting Michaelis constants for aldehyde and NADH respectively. The results of initial rate studies plotted according to Lineweaver-Burk plot are shown in Fig. 12 for furfural and NADH and in Fig. 14. for acetaldehyde and NADH. The slopes and intercepts obtained in these plots are replotted and shown in Figs. 13 and 15 respectively. The estimated kinetic constants of V_{mf}, V_{mr}, K_a, K_b, K_p, K_q, K_{ia} and K_{iq} are summarized in Table 2.

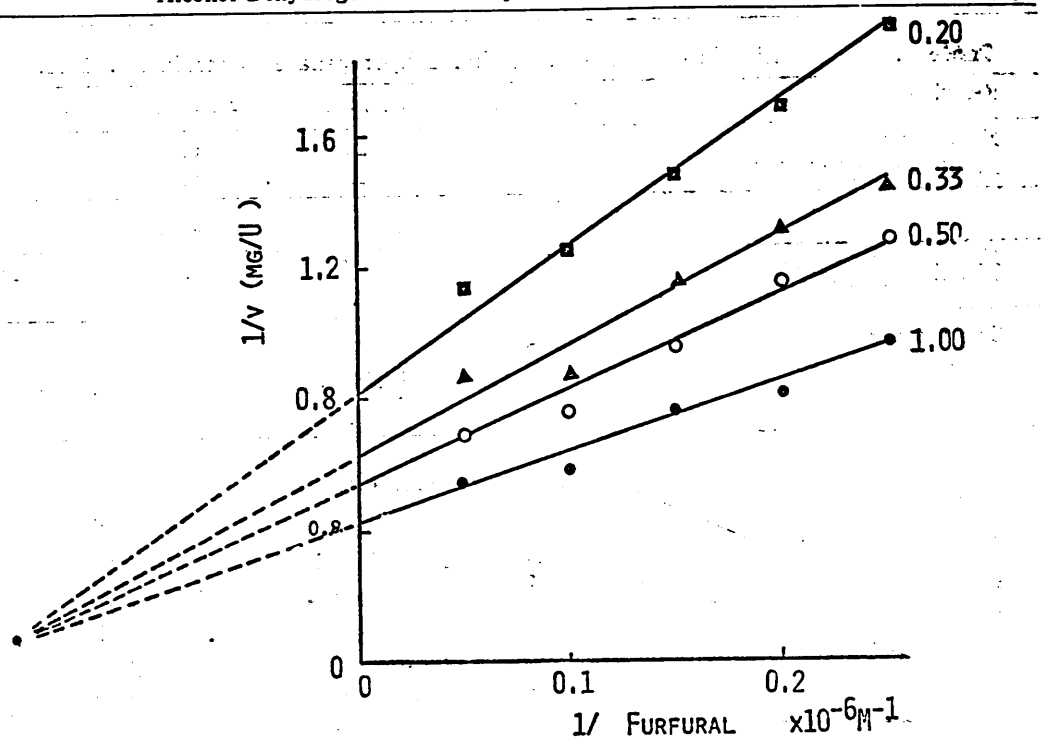

Fig. 12. Lineweaver-Burk plots for the reduction of furfural with alcohol dehydrogenase of strain No. 1001. The reaction was carried out at 30°C in 10mM potassium phosphate buffer (pH7.4) and NADH and furfural as specified. The NADH concentration was 0.20, 0.33, 0.50 and 1.00 x10⁻⁴M as specified in the figure.

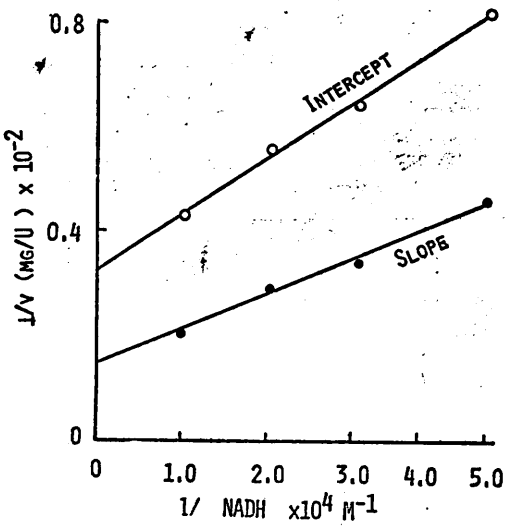

Fig. 13. Replots of the slopes and intercepts is the Lineweaver-Burk plots shown in Fig. 12. against the reciplocal of NADH concentrations.

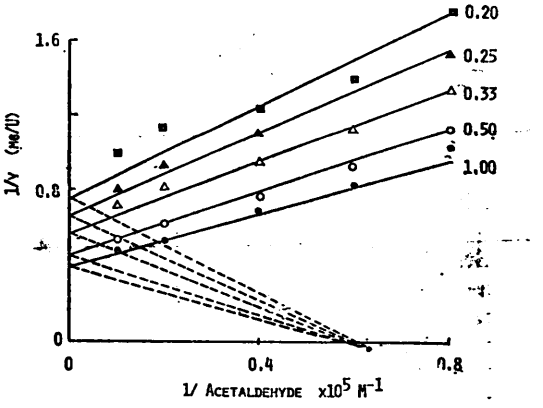

Fig. 14. Lineweaver-Burk plots for the reduction of acetaldehyde with alcohol dehydrogenase of strain No. 1001. The reaction conditions were essentially same as in Fig. 12 except acetaldehyde was used instead of furfural. NADH. NADH concentrations used were 0.20, 0.25, 0.33, 0.50 and 1.00 x10⁻⁴M as specified in the figure.

Table 2. Kinetic constants ot alcohol dehydrogenase of strain No. 1001.

Reaction	V_{mf}(U/mg)	K_a(μM)	K_b(μM)	K_{ia}(μM)
NAD+-Ethanol	71	28	680	56
NAD+-Furfuryl alcohol	27	19	560	85
	V_{mr}(U/mg)	K_p((μM)	K_q(μM)	K_{iq}(μM)
NADH-Acetaldehyde	330	17	28	26
NADH-Furfural	310	4.8	30	39

Tho notations of the reactants and products are as follows

A(NAD+) ↓ B(alcohol) ↓ P(aldehyde) ↑ Q(NADH) ↑

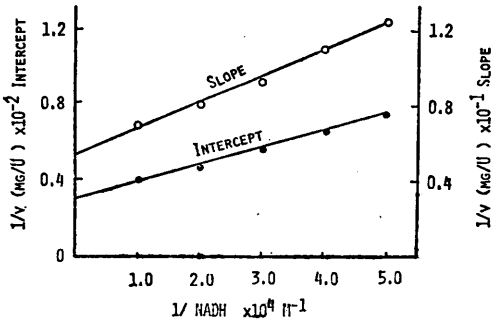

Fig. 15. Replots of the slope and intercepts of Lineweaver-Burk plots shown in Fig 14. against the reciprocal of NADH concentrations.

Substrate specificity

To compare the substrate specificity range of the alcohol dehydrogenase of strain No. 1001 to that of other origin, NAD+ reduction in the presence of various alphatic and aromatic alcohols was studied and listed in Table 3. As comparisons substrate specificities of yeast and horse liver alcohol dehydrogenases were studied at the same reaction conditions. From the table it is evident that yeast alcohol dehydrogenase is not active on furfuryl alcohol and that of horse liver alcohol dehydrogenase has only 1/40 activity toward furfuryl alcohol

Table 3. The substrate specificities of alcohol dehydrogenases of strain No. 1001, yeast and horse liver: activity toward alcohols.

Alcohols	Strain No. 1001		Yeast		Horse liver	
	Activity (U/mg)	Relative activity %	Activity (U/mg)	Relative activity %	Activity (U/mg)	Relative activity %
Ethanol	54	1.0	148	1.0	0.56	1.0
n-Propanol	49	0.9	25	0.17	1.00	1.79
n-Butanol	45	0.8	8	0.05	1.04	1.86
n-Pentanol	38	0.7	5	0.03	0.40	0.71
n-Hexanol	33	0.6	3	0.02	0.40	0.68
n-Octanol	29	0.5	7	0.05	0.40	0.68
iso-Propanol	0	0	0	0	0	0
iso-Butanol	6	0.1	0	0	1.06	1.89
iso-Pentanol	4	0.1	0	0	0.44	0.79
sec-Butanol	5	0.1	0	0	0.28	0.50
2-Methyl hexanol	0	0	0	0	1.96	3.50
Allyl alcohol	86	1.6	124	0.84	1.12	2.00
Benzyl alcohol	4	0.1	0	0	0	0
Furfuryl alcohol	20	0.4	0	0	0.28	0.50

Reaction mixture contained 50mM Tris-HCl buffer pH 8.5, 100 μM NAD+ and 10mM substrate.

Table 4. The substrate specificities of alcohol dehydrogenase of strain No. 1001, Yeast and horse liver: Activity toward aldehyde.

Aldehyde	Strain No. 1001		Yeast		Horse liver	
	Activity (U/mg)	Relative activity %	Activity (U/mg)	Relative activity %	Activity (U/mg)	Relative activity %
Formaldehyde	70	0.28	0	0	3.5	0.14
Acetaldehyde	250	1.00	896	1.00	24.9	1.00
Propionaldehyde	183	0.73	320	0.36	26.7	1.07
n-Butylaldehyde	116	0.46	24	0.03	10.6	0.43
iso-Butylaldehyde	4	0.02	0	0	0.6	0.02
Glyoxal	7	0.03	0	0	0.5	0.02
Glutalaldehyde	93	0.37	10.3	0.01	23.2	0.93
Chroral hydrate	3	0.01	0	0	0.2	0
Benzaldehyde	98	0.31	0	0	13.1	0.53
Furfural	240	0.96	9	0.01	13.3	0.53

The reaction mixture contained 100mM Potassium phosphate buffer pH 7.4 100μM NADH and 5mM substrate.

in mg protein base even though relative activity toward ethanol is enough high.

In Table 4. substrate specificities of the reverse reaction of alcohol dehydrogenase of strain No. 1001, yeast and horse liver are listed. Furfural is one of the best substrate for the enzyme of strain No. 1001, but yeast alcohol dehydrogenase is inactive and horse liver enzyme again had little activity to all aldehyde tested in specific activity in mg protein base.

Discussion

There are good reasons to believe that alcohol dehydrogenases of al lorganisms evolved from the same origin. Yeast and horse liver alcohol dehydrogenases have been most extensively studied and their amino acid sequences have been decided recently (6-8, 13, 40).

In a comparative study of these two enzymes (41), it was concluded that the overall positional identity is 25% and that the longest apparent corrrespond is 21 residues. So, alcohol dehydrogenases studied in this thesis may also be on the same line of the correlation between alcohol dehydrogenases of yeast and horse liver. Horse liver alcohol dehydrogenase is a dimer enzyme of molecular weight of 83, 000(46) and it is most active on n-butanol amonng n-alcohols. It has a wide substrate specificity range and very low specific activity compared to yeast alcohol dehydrogenase. These characteristics could be reasonable to consider its role in mammalian liver to detoxificate higher alcohols produced by anaerobic bacteria in the digestive organs. Yeast alcohol dehydrogenase is characteristic to have very high specific activity to ethanol and relatively narrow substrate specificity range. This might be a result of that yeast alcohol dehydrogenase have been selected as a catalyst of alcohol fermentation during evolution of the enzyme molecule. Yeast alcohol dehydrogenase has 5% activity on n-butanol as that of ethanol.

In this thesis two alcohol dehydrogenases of different origin which are active on furfuryl alcohol were purified and studied. These two enzymes had very similar substrate specificity ranges. Both enzymes had almost same acti-

vities on ethanol, n-propanol, n-butanol and on n-pentanol, and had 35-40% activity on furfuryl alcohol as that on ethanol. These facts may reflect the selection standard during molecular evolution that is active on furfuryl alcohol essentially lead to this substrate range.

In comparison of the primary structures on yeast and horse liver alcohol dehydrogenases, both molecules have its own deletion and insertion segments, but high similarity was found in active site region and dissimilar region in loop segements. The role of such loop region is not known yet but may reflect the substrate specificity which might be decided by its stereochemical structures.

In Table 4 substrate specificity of known alcohol dehydrogenases are listed. Only horse liver enzyme is known to be active on furfury alcohol, and other enzymes are negative or no information is available. So, in these experiments the existence of alcohol dehydrogenase active on furfuryl alcohol has been established.

Some examples are known that dehydrogenase having more specific substrate ranges including common substrates as alcohol dehydrogenases (43-45). Harada st al (43) have reported 2-alkyne-1-ol dehydrogenase having molecular weight of more than 500,000. This enzyme is not active on ethanol but very active on 2-butyne-1-ol and named as 2-alkyne-1-ol dehydrogenases. The enzyme still has activity on n-propanol as a common substrate with alcohol dehydrogenase. 1, 2-propandiol dehydrogenase studied by Fukui et al (44) has an intermediate substrate specificity range between alcohol dehydrogenase of horse liver and 2-alkyne-1-ol dehydrogenase. It has 5% activity on ethanol of that of on n-propanol and is very active on 1,2-propandiol which is a common substrate with 2-alkyne-1-ol dehydrogenase. So taking the substrate specificity range of alcohol dehydrogenase of horse liver as a prototype, the yeast enzyme is enhanced its action on ethanol and lost its activity on various higher alcohols. 1,2-propandiol dehydrogenase have evolved to obtain action on the diol and lost most of its action on ethanol. This tendency is more remarkable in 2-alkyne-1-ol dehydrogenase which activity on ethanol is completely lost. From the standpoint of alcohol dehydrogenase evolution the enzyme studied in this thesis, it is concluded that the activity on furfuryl alcohol does not require such drastic molecular evolutional changes. Their substrate specificity range is the same degree as horse liver enzyme, though their activities are 100 times higher than that of horse liver enzymes.

Enzymes are classified by their substrate specificity differences, and not by the primary structural differences. The problem is extream evolution of enzme molecule results in lack of activity on standard substrate such as ethanol in case of alcohol dehydrogenase 2-Alkyne-1-ol dehydrogenase may be one of the cases.

Classification of such enzyme should be alcohol dehydrogenase in systematic evolutional nomenclature but could be quite a new enzyme by a trivial substrate specificify nomenclature. The disadvantage of the systematic evolutional nomenclature is that the name of the enzyme can not be made before its primary structure decision. So, these two nomenclature systems should be interact to made suitable nomenclature system.

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